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APPLICANT: Lothar Eggeling *et al.*

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EXAMINER: C. Fronda

FOR: "Method for Microbially Producing L-Valine"

**MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450**

Dear Sir,

SUBMISSION OF TRANSLATION OF PRIORITY APPLICATION

Application Serial No. 09/914,006 is a national phase application of PCT/EP00/01450, which claims priority from DE 199 07 567.0 filed February 22, 1999. In accordance with 37 CFR 1.55, Applicants submit herewith an English language translation of priority application DE 199 07 567.0 and statement that the translation is an accurate translation of the priority application.

No fee is believed to be due with this paper. The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 03-2775, under Order No. 05899-00013-US. A duplicate copy of this paper is enclosed.

Dated: December 13, 2007

Respectfully submitted,

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TRANSLATION

THE FEDERAL REPUBLIC OF GERMANY



Priority Certificate DE 199 07 567.0 on the Filing of a Patent Application

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Date of filing: February 22, 1999
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52428 Jülich, Germany (DE)
Title: Method for the microbial production of L-valine
IPC: C 12 N 1/21, C 12 P 13/04

The appended documents are a true and precise reproduction of the parts of the documentation pertaining to this patent application filed on February 22, 1999, irrespective of any color deviations that may exist owing to the copying process.

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I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true.

In witness whereof I sign,

December 6, 2007

Date

Elise Duvekot

Signature of translator



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CERTIFICATE OF VERIFICATION OF TRANSLATION

I, Elise Duvekot, a citizen of the United States of America, hereby certify that I am fully familiar with the German and English languages and that I am capable of translating from German into English. To the best of my knowledge and ability, the foregoing pages constitute an accurate and complete translation of the copy before me in the German language of German Patent Application DE 199 07 567.0 titled "Verfahren zur mikrobiellen Herstellung von L-Valin".

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December 6, 2007

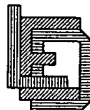
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Method for the microbial production of L-valine

10 The present invention relates to a method for the microbial production of L-valine according to Claims 1 to 13 as well as to transformed cells or microorganisms according to Claim 14 that can be employed in the method.

The amino acid L-valine is a commercially significant product that is used in animal
15 nutrition, in human nutrition and in medicine. Consequently, there is a general interest in providing improved methods for the production of L-valine.

Valine can be produced by chemical synthesis or else by biotechnology through the fermentation of suitable microorganisms in suitable nutrient solutions. The advantage
20 of the biotechnological production by means of microorganisms lies in the formation of the correct stereoisomeric form, namely, the L-form of valine free of D-valine.

Several types of bacteria such as, for example, *Escherichia coli*, *Serratia marcescens*, *Corynebacterium glutamicum*, *Brevibacterium flavum* or *Brevibacterium lacto-*
25 *fermentum* can produce L-valine in a nutrient solution containing glucose. U.S. Pat. No. 5,658,766 shows that, with *Escherichia coli*, an increased formation of L-valine can be achieved through mutation in the aminoacyl-tRNA synthetase. WO 96 06926 also shows that, by means of lipolic acid auxotrophy, an increase in the formation of L-valine can be achieved with *Escherichia coli*. European patent application

EP 0 694 614 A1 describes strains of *Escherichia coli* that are resistant to α -ketobutyric acid and that produce L-valine, L-isoleucine or L-leucine in a nutrient solution containing glucose.

- 5 In EP 0 477 000, it is shown that the mutagenesis of *Corynebacterium* or *Brevibacterium* and the selection for valine resistance can improve the formation of L-valine. In the same European publication, it is also shown that an improved formation of L-valine can be achieved through the selection of *Corynebacterium* or *Brevibacterium* for resistance to various pyruvate analogs such as β -fluoropyruvate,
10 β -chloropyruvate, β -mercaptopyruvate or trimethyl pyruvate. Nakayama *et al.* (Nakayama *et al.*, 1961, J. Gen. Appl. Microbiol. Jpn.) have disclosed that auxotrophies introduced by undirected mutations can lead to improved accumulation of L-valine.

- Moreover, European patent application EP 0 356 739 A1 shows that the formation of
15 L-valine is improved through the amplification of the DNA region that codes for acetohydroxy acid synthase (*ilvBN*, also see Figure 1) by means of the plasmid pAJ220V3.

- It is the objective of the present invention to provide new foundations for the micro-
20 bial production of L-valine, especially by means of coryneform bacteria.

- This objective is achieved according to the invention in that the dihydroxy acid dehydratase-(*ilvD*)-activity and/or the *ilvD*-gene expression in a microorganism is intensified. As an alternative to this or in combination with this, the acetohydroxy
25 acid synthase-(*ilvBN*)-activity and isomeroreductase-(*ilvC*)-activity and/or the *ilvBNC*-gene expression in a microorganism are intensified. For the method according to the invention, microorganisms can be additionally used in which the activity of at least one enzyme participating in a metabolic pathway that reduces L-valine formation is weakened or switched off. Thus, the methods according to the invention

preferably make use of microorganisms having a defective mutation in the threonine dehydratase-(ilvA-) gene and/or having a defective mutation in one or more genes of the pantothenate synthesis.

- 5 The terms “valine” or “L-valine” as employed in the claimed invention refer not only to the free acid but also to the salt thereof such as, for instance, calcium, sodium, ammonium or potassium salt.

- 10 The term “intensification” describes the increase in the intracellular activity of the cited ilvD, ilvB, ilvN and ilvC enzymes. In order to increase the enzyme activity, especially the endogenous activity in the microorganism is increased. The enzyme activity can be increased, for example, in that a greater substrate reaction occurs by changing the catalytic center or in that the effect of enzyme inhibitors is eliminated. Greater enzyme activity can also be brought about by increasing the enzyme synthe-
- 15 sis, for instance, through gene amplification or by switching off factors that repress enzyme biosynthesis. The endogenous enzyme activity is increased according to the invention preferably through mutation of the corresponding endogenous gene. Such mutations can be generated either in an undirected manner according to classic methods such as, for example, with UV irradiation or with chemicals that trigger mutation,
- 20 or else in a directed manner by means of genetic-engineering methods such as deletion(s), insertion(s) and/or nucleotide exchange(s).

- The gene expression is intensified according to the invention preferably by increasing the number of gene copies. For this purpose, the gene or genes is/are incorporated
- 25 into a gene construct or into a vector that preferably contains regulatory gene sequences associated with the genes, especially those that intensify the gene expression. Subsequently, a microorganism, preferably *Corynebacterium glutamicum*, is transformed with the corresponding gene constructs.

It was ascertained that L-valine is produced in an improved manner through the intensified expression of the valine biosynthesis *ilvD* gene from *Corynebacterium glutamicum* – which codes for the enzyme dihydroxy acid dehydratase. According to the invention, in addition to the intensified expression of this gene, the intensified expression of the *ilvBN* genes – which code for the enzyme acetohydroxy acid synthase – as well as of the *ilvC* gene – which codes for the enzyme isomeroreductase – brings about improved L-valine formation in *Corynebacterium glutamicum*. Further improvement of the formation of L-valine in *Corynebacterium glutamicum* is achieved by over-expression of all of the cited genes. The genes or gene constructs can be present in the host organism either in plasmids having a different number of copies or else they can be integrated and amplified in the chromosome.

A further increase in the gene expression can be achieved, as an alternative to or in combination with an increase in the number of gene copies, by intensifying regulatory factors that have a positive influence on the gene expression. For instance, the regulatory elements can be intensified on the transcription level especially by using intensified transcription signals. Moreover, the promoter and regulation region that is located upstream from the structural gene can be mutated. Expression cassettes that are incorporated upstream from the structural gene function in the same manner. Through inducible promoters, it is additionally possible to increase the expression over the course of the fermentative formation of L-valine. Aside from this, however, the translation can also be intensified by improving, for example, the stability of the m-RNA. Furthermore, genes can be employed that code for the corresponding enzyme having a high activity. As an alternative, an over-expression of the appertaining genes can also be achieved by changing the media composition and the culture management. A person skilled in the art can find instructions for this, among other places, in Martin *et al.* (Bio/Technology 5, 137-146 (1987)), in Guerrero *et al.* (Gene 138, 35-41 (1994)), in Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), in Eikmanns *et al.* (Gene 102, 93-98 (1991)), in European patent specifica-

tion EP 0 472 869, in U.S. Pat. No. 4,601,893, in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)), in Reinscheid *et al.* (Applied and Environmental Microbiology 60, 126-132 (1994)), in LaBarre *et al.* (Journal of Bacteriology 175, 1001-1007 (1993)) and in international patent application WO 96/15246.

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All conceivable combinations of the above-mentioned measures are also a possibility for the intensification of the gene expression.

Microorganisms that can be used in the method according to the invention are capable of producing L-valine from glucose, saccharose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerin and ethanol. They can be gram-positive bacteria, for instance, belonging to the *Bacillus* genus or coryneform bacteria of the above-mentioned *Corynebacterium* genus or else *Arthrobacter*. When it comes to the *Corynebacterium* genus, special mention was already made of the *Corynebacterium glutamicum* species, which is known in technical circles for its ability to form amino acids. This species includes wild type strains such as, for instance, *Corynebacterium glutamicum* ATCC13032, *Brevibacterium flavum* ATCC14067, *Brevibacterium lactofermentum* ATCC13869, *Brevibacterium thiogenitalis* ATCC19240, *Corynebacterium melassecola* ATCC17965 and others.

20

In order to isolate the *ilvD* gene of *Corynebacterium glutamicum* or other genes, first of all, a gene library is created. The creation of gene libraries has been described in generally known textbooks and manuals. Examples of these are the textbook by Winnacker: Gene und Klone, Eine Einführung in die Gentechnologie [Genes and clones, an introduction to genetic engineering] (published by Verlag Chemie, Weinheim, Germany, 1990) or the manual by Sambrook *et al.*: Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989). A known gene library is that of the *E. coli* K-12 strain W3110, which was created in λ -vectors by Kohara *et al.* (Cell 50, 495-508 (1987)). Bathe *et al.* (Molecular and General Genetics, 252: 255-256,

25

1996) describe a gene library of *Corynebacterium glutamicum* ATCC13032, which was created using the cosmid vector SuperCos I (Wahl *et al.*, 1987, Proceedings of the National Academy of Sciences, U.S.A., 84: 2160-2164) in the *E. coli* K-12 NM554 (Raleigh *et al.*, 1988, Nucleic Acids Research 16: 1563-1575). Plasmids such as pBR322 (Bolivar, Life Sciences, 25, 807-818 (1979)) or pUC19 (Norrander *et al.*, 1983, Gene, 26: 101-106) can also be used to create a gene library of *Corynebacterium glutamicum* in *Escherichia coli*. Plasmids such as pJC1 (Cremer *et al.*, Mol. Gen. Genet. (1990) 220: 3221-3229) or pECM2 (Jäger *et al.*, J. Bacteriol. (1992) 174: 5462-5465) can be used to create a gene library of *Corynebacterium glutamicum* in *Corynebacterium glutamicum*. Suitable hosts are especially those bacteria strains that are restriction-defective and recombination-defective. An example of this is the *Escherichia coli* DH5amcr strain, which was described by Grant *et al.* (Proceedings of the National Academy of Sciences U.S.A., 87 (1990) 4645-4649), or the *Corynebacterium glutamicum* R127 strain, which was isolated by Liebl *et al.* (FEMS Lett. (1989) 65: 299-304).

The gene library is subsequently incorporated into an indicator strain by transformation (Hanahan, Journal of Molecular Biology 166, 557-580, 1983) or by electroporation (Tauch *et al.*, 1994, FEMS Microbiological Letters, 123: 343-347). The indicator strain is characterized by the fact that it has a mutation in the gene of interest, which brings about a detectable phenotype, for example, an auxotrophy. The indicator strains or mutants can be obtained from published sources or strain collections or else, if need be, have to be produced for that specific purpose. Within the scope of the present invention, the *Corynebacterium glutamicum* mutant R127/7 was isolated that is defective in the *ilvD* gene that codes for the dihydroxy acid dehydratase. After the transformation of the indicator strain such as, for instance, the *ilvD* mutant R127/7, with a recombinant plasmid that carries the gene of interest such as, for instance, the *ilvD* gene and after the expression thereof, the indicator strain becomes prototrophic relative to the applicable property such as, for example, the need for L-valine.

The gene or DNA fragment isolated in this manner can be characterized by determining the sequence, as described, for example, in Sanger *et al.* (Proceedings of the National Academy of Sciences of the United States of America U.S.A., 74: 5463-5467, 1977). Subsequently, the degree of identity to known genes that are contained in databases such as, for instance, GenBank (Benson *et al.*, 1998, Nucleic Acids Research, 26: 1-7), can be analyzed using published methods (Altschul *et al.*, 1990, Journal of Molecular Biology 215: 403-410).

10 In this manner, the DNA sequence of *Corynebacterium glutamicum* that codes for the ilvD gene was obtained which, as SEQ ID NO. 1, is an integral part of the present invention. Furthermore, the amino acid sequences of the corresponding enzymes were used to derive from the DNA sequence on hand by means of the above-mentioned methods. The resulting amino acid sequence of the ilvD gene product, namely of
15 dihydroxy acid dehydratase, is designated as SEQ ID NO. 2.

The gene characterized in this manner can be subsequently brought to expression either individually or in combination with others in a suitable microorganism. A known method to express or over-express genes consists of amplifying said genes by
20 means of plasmid vectors which can moreover be provided with expression signals. Suitable plasmid vectors are those that can replicate in the corresponding microorganisms. In the case of *Corynebacterium glutamicum*, possibilities are, for instance, the vectors pEKEx1 (Eikmanns *et al.*, Gene 102: 93-98 (1991)) or pZ8-1 (European patent specification 0 375 889) or pEKEx2 (Eikmanns *et al.*, Microbiology 140:
25 1817-1828 (1994) or pECM2 (Jäger *et al.*, Journal of Bacteriology 174(16): 5462-5465 (1992)). Examples of such plasmids are pJC1ilvD, pECM3ilvBNCD, and pJC1ilvBNCD. These plasmids are *Escherichia coli*/*Corynebacterium glutamicum* shuttle vectors that carry the ilvD gene or the ilvD gene together with the genes ilvB, ilvN and ilvC.

The inventors have also found that the intensification of the gene individually or in combination with the *ilvB*, *ilvN* and *ilvC* genes has an advantageous effect in those microorganisms that have a reduced synthesis of the amino acid L-isoleucine. This
5 reduced synthesis can be achieved through the deletion of the *ilvA* gene that codes for the L-isoleucine synthesis specific enzyme, namely, threonine dehydratase.

The deletion can be carried out by directed recombinant DNA techniques. Using these methods, for example, the *ilvA* gene that codes for the threonine dehydratase
10 can be deleted in the chromosome. Suitable methods for this are described in Schäfer *et al.* (Gene (1994) 145: 69-73) or in Link *et al.* (Journal of Bacteriology (1998) 179: 6228-6237). It is also possible for only parts of the gene to be deleted, or else for mutated fragments of the threonine dehydratase gene to be exchanged. In this manner, a loss of the threonine dehydratase activity is achieved by deletion. An example
15 of such a mutant is the *Corynebacterium glutamicum* strain ATCC13032 Δ *ilvA*, that carries a deletion in the *ilvA* gene.

The inventors have also found that the intensification of the *ilvD*, *ilvB*, *ilvN* and *ilvC* genes in another combination with the reduced synthesis of D-pantothenate, preferably in combination with further deletion of the *ilvA* gene, has an advantageous effect
20 on L-valine formation in microorganisms, for example, by deletions in the *panB* and *panC* genes. The reduced D-pantothenate synthesis can be achieved by weakening or switching off the corresponding biosynthesis enzymes or their activities. Possibilities for this are, for example, the enzymes ketopantoate hydroxymethyl transferase (EC
25 2.1.2.11), ketopantoate reductase, pantothenate ligase (EC 6.3.2.1) and aspartate decarboxylase (EC 4.1.1.11). One way to switch off or weaken enzymes and their activities includes mutagenesis methods.

These include undirected methods that use chemical reagents such as, for example, N-methyl-N-nitro-N-nitrosoguanidine, or else UV radiation for the mutagenesis, with a subsequent search for the desired microorganisms for their need for D-pantothenate. Methods for causing mutations and searching for mutants are generally known and
 5 can be found, among other places, in Miller (A Short Course in Bacterial Genetics, A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria (Cold Spring Harbor Laboratory Press, 1992)) or in the manual titled "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., U.S.A., 1981).

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Moreover, these include directed recombinant DNA techniques. Using these methods, for example, the genes panB, panC, panE and panD that code for ketopantoate hydroxymethyl transferase, pantothenate ligase, ketopantoic acid reductase or aspartate decarboxylase can be deleted in the chromosome individually or else jointly .
 15 Methods suitable for this are described in Schäfer *et al.* (Gene (1994) 145: 69-73) or in Link *et al.* (Journal of Bacteriology (1998) 179: 6228-6237). It is also possible for only parts of the gene to be deleted, or else for mutated fragments of ketopantoate hydroxymethyl transferase, pantothenate ligase, ketopantoic acid reductase and aspartate decarboxylase to be exchanged. Thus, a loss or a reduction of the enzyme
 20 activity in each case is achieved through deletion or exchange. An example of such a mutant is the *Corynebacterium glutamicum* strain ATCC13032 Δ panBC, which carries a deletion in the panBC operon.

The microorganisms produced according to the invention can be cultivated continuously or discontinuously in a batch process (batch cultivation) or in a fed batch (feed
 25 method) or in a repeated fed batch method (repetitive feed method) for purposes of L-valine production. A summary of known cultivation methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Process biotechnology 1. Introduction to bioprocess technology] (published by Gus-

tav Fischer Verlag, Stuttgart, Germany, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and peripheral devices] (published by Vieweg Verlag, Braunschweig / Wiesbaden, Germany, 1994)).

- 5 The culture medium to be used has to meet the demands of the microorganisms in question in a suitable manner. Descriptions of culture media of various microorganisms are described in the manual titled "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., U.S.A., 1981). Sources of carbon that can be used include sugar and carbohydrates such as, for
10 example, glucose, saccharose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats such as, for instance, soy oil, sunflower oil, peanut oil and coconut oil, fatty acids such as, for example, palmitic acid, stearic acid and linoleic acid, alcohols such as, for example, glycerin and ethanol and organic acids such as, for instance, acetic acid. These substances can be used individually or in a mixture.
15 Sources of nitrogen that can be used include organic compounds containing nitrogen such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soybean flour and urea or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. These nitrogen sources can be used individually or as mixtures. Sources of phosphorus that
20 can be used include potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding salts containing sodium. The culture medium also has to contain salts of metals such as, for example, magnesium sulfate or iron sulfate that are needed for the growth. Finally, essential growth promoters such as amino acids and vitamins can be used in addition to the above-mentioned substances. The above-
25 mentioned feed materials can be fed into the culture in the form of a one-time batch or can be added in a suitable manner during the cultivation.

In order to control the pH of the culture, alkaline compounds such as sodium hydroxide, potassium hydroxide, ammonia or acidic compounds such as phosphoric

acid or sulfuric acid are employed in a suitable manner. In order to regulate the foam formation, anti-foaming agents such as, for example, fatty acid polyglycol esters can be used. In order to maintain the stability of plasmids, suitable selectively acting substances, e.g. antibiotics, can be added to the medium. In order to maintain aerobic
5 conditions, oxygen or gas mixtures containing oxygen such as, for instance, air, can be fed into the culture. The temperature of the culture is normally between 20°C and 50°C [68°F and 122°F] and preferably between 25°C and 45°C [77°F and 113°F]. The culture is maintained until a maximum of L-valine has been formed. This objective is normally achieved within 10 to 160 hours.

10 The concentration of formed L-valine can be determined using known methods (Jones and Gilligan (1983) Journal of Chromatography 266: 471-482).

The invention will be explained in greater detail with reference to the following
15 embodiments.

Example 1: Cloning, sequencing and expression of the *ilvD* gene from *Corynebacterium glutamicum* that codes for dihydroxy acid dehydratase

20 **1. Isolation of an *ilvD* mutant of *Corynebacterium glutamicum***

The *Corynebacterium glutamicum* strain R127 (Haynes 1989, FEMS Microbiology
25 Letters 61: 329-334) was mutagenized with N-methyl-N-nitro-N-nitrosoguanidine (Sambrook *et al.*, Molecular cloning. A laboratory manual (1989) Cold Spring Harbor Laboratory Press). For this purpose, 5 ml of a *Corynebacterium glutamicum* culture that was grown overnight were mixed with 250 µl of N-methyl-N-nitro-N-nitrosoguanidine (5 mg/ml of dimethyl formamide) and incubated for 30 minutes at 30°C

[86°F] and 200 rpm (Adelberg 1958, Journal of Bacteriology 76: 326). The cells were subsequently washed twice with sterile NaCl solution (0.9%). Through replica plating on CGXII minimal medium plates with 15 g/l of agar (Keilhauer *et al.*, Journal of Bacteriology 175: 5595-5603), mutants were isolated that only grew under the addition of L-valine, L-isoleucine and L-leucine (0.1 g/l each).

The enzyme activity of the dihydroxy acid dehydratase was determined in the raw extract of these mutants. For this purpose, the clones were cultivated in 60 ml of LB medium and centrifuged off in the exponential growth phase. The cell pellet was washed once with 0.05 M potassium phosphate buffer and resuspended in the same buffer. The cell disruption was done by means of a 10-minute ultrasound treatment (Branson-Sonifier W-250, Branson Sonic Power Co, Danbury, U.S.A.). Subsequently, the cell components were separated by means of a 30-minute centrifugation at 13,000 rpm and at 4°C [39.2°F] and the supernatant was used as the raw extract for the enzyme test. The reaction batch of the enzyme test contained 0.2 ml of 0.25 M Tris/HCl, pH 8, 0.05 ml of raw extract, and 0.15 ml of 65 mM α,β -dihydroxy- β -methyl valerate. The test batches were incubated at 30°C [86°F] and, after 10, 20 and 30 minutes, 200 μ l-samples were taken and their ketomethyl valerate concentration was determined by means of HPLC analysis (Hara *et al.* 1985, Analytica Chimica Acta 172: 167-173). As Table 1 shows, the strain R127/7 does not display any dihydroxy acid dehydratase activity, in contrast to which the isomeroreductase and aceto-hydroxy acid synthase activities are still present as further enzymes of the synthesis of the branched-chain amino acids.

Table 1

Specific activities (μ mol/min and mg of protein) of valine biosynthesis enzymes in *Corynebacterium glutamicum* strains

Strain	Dihydroxy acid dehydratase	Isomeroeductase	Acetohydroxy acid synthase
R127	0.003	0.05	0.07
R127/7	0.000	0.06	0.09

2. Cloning the *ilvD* gene of *Corynebacterium glutamicum*

5 Chromosomal DNA from *Corynebacterium glutamicum* R127 was isolated, as described in Schwarzer and Pühler (Bio/Technology 9 (1990) 84-87). This was cleaved with the restriction enzyme Sau3A (Boehringer Mannheim, Germany) and fractionated by means of saccharose density gradient centrifugation (Sambrook *et al.*, Molecular cloning. A laboratory manual (1989) Cold Spring Harbor Laboratory Press). The fraction with the fragment size range from about 6 kb to 10 kb was used for the ligation with the vector pJC1 (Cremer *et al.*, Molecular and General Genetics 220 (1990) 478-480). For this purpose, the vector pJC1 was linearized with BamHI and dephosphorylated. Five ng of this were ligated with 20 ng of the above-mentioned fraction of the chromosomal DNA and used to transform the mutant

10 R127/7 by means of electroporation (Haynes and Britz, FEMS Microbiology Letters 61 (1989) 329-334). The transformants were tested for their capability to grow on CGXII agar plates without the addition of branched-chain amino acids. Of the over 5000 tested transformants, after replica plating and a two-day incubation at 30°C [86°F], 8 clones grew on minimal medium plates. Plasmid preparations were made

15 from these clones as described in Schwarzer *et al.* (Bio/Technology (1990) 9: 84-87). Restriction analyses of the plasmid DNA showed that the same plasmid, referred to as pRV below, was present in all 8 clones. The plasmid has an insert of 4.3 kb and was tested by means of retransformation for its ability to complement the *ilvD* mutant R127/7. Through subcloning, the region responsible for the complementation of the

20 mutant R127/7 was limited to a 2.9 ScaI/XhoI fragment (Figure 2).

3. Sequencing the ilvD gene

5 The nucleic acid sequencing of the 2.9 ScaI/XhoI fragment was carried out by the dideoxy chain termination method of Sanger *et al.* (Proceedings of the National Academy of Sciences of the United States of America U.S.A. (1977) 74: 5463-5467). The Auto-Read Sequencing kit was used (Amersham Pharmacia Biotech, Uppsala, Sweden). The gel electrophoretic analysis was carried out with an automatic laser
10 fluorescence sequencing device (A.L.F.) made by Amersham Pharmacia Biotech (Uppsala, Sweden). The nucleotide sequence obtained was analyzed with the HUSAR program package (Release 4.0, EMBL, Cambridge, GB). The nucleotide sequence is designated as ID SEQ NO 1. The analysis showed an open reading frame of 1836 base pairs that was identified as an ilvD gene and that codes for a polypeptide of 612
15 amino acids that is designated as ID SEQ NO 2.

4. Expression of the ilvD gene

20 The plasmid pRV was digested with the restriction enzymes ScaI and XhoI, corresponding to the information from the manufacturer of the restriction enzymes (Roche, Boehringer Mannheim, Germany). Subsequently, the 2.9-kb ilvD fragment was isolated by means of an ion exchanger column (Quiagen, Hilden, Germany). The overhanging end of the XhoI section of the isolated fragment was filled up with Klenow
25 polymerase. The vector pJC1 (Cremer *et al.*, Mol. Gen. Genet. (1990)220: 478-480) was PstI-cut, likewise treated with Klenow polymerase, and subsequently the fragment and the vector were ligated. Using the ligation batch, the *E. coli* strain DH5amcr (Grani *et al.*, Proceedings of the National Academy of Sciences of the United States of America U.S.A., 87 (1990) 4645-4649) was transformed (Hanahan,

Journal of Molecular Biology 166 (1983) 557-580). Through plasmid preparations of clones (Sambrook *et al.*, Molecular cloning. A laboratory manual (1989) Cold Spring Harbor Laboratory Press), a clone was identified that contained the recombinant plasmid pJC1ilvD. With this plasmid, *Corynebacterium glutamicum* ATCC13032

5 was transformed by means of electroporation as described in Haynes *et al.* (1989, FEMS Microbiol. Lett. 61: 329-334). Subsequently, the dihydroxy acid dehydratase activity coded by ilvD was determined for *Corynebacterium glutamicum* ATCC13032 pJC1 and *Corynebacterium glutamicum* ATCC13032 pJC1ilvD. For this purpose, the clones were cultivated in 60 ml of LB medium and centrifuged off in

10 the exponential growth phase. The cell pellet was washed once with 0.05 M potassium phosphate buffer and resuspended in the same buffer. The cell disruption was carried out by means of a 10-minute ultrasound treatment (Branson-Sonifier W-250, Branson Sonic Power Co, Danbury, U.S.A.). Subsequently, the cell components were separated by means of a 30-minute centrifugation at 13,000 rpm and at 4°C [39.2°F]

15 and the supernatant was used as the raw extract for the enzyme test. The reaction batch of the enzyme test contained 0.2 ml of 0.25 M Tris/HCl, pH 8, 0.05 ml of raw extract, and 0.15 ml of 65 mM α,β -dihydroxy- β -methyl valerate. The test batches were incubated at 30°C [86°F] and, after 10, 20 and 30 minutes, 200 μ l-samples were taken and their ketomethyl valerate concentration was determined by means of HPLC

20 analysis (Hara *et al.* 1985, Analytica Chimica Acta 172: 167-173). As Table 2 shows, the strain *Corynebacterium glutamicum* ATCC13032 pJC1ilvD exhibits an elevated dihydroxy acid dehydratase activity in comparison to the control strain.

Table 2

25

Specific activity (μ mol/min and mg of protein) of dihydroxy acid dehydratase in *Corynebacterium glutamicum* ATCC13032

Plasmid	dihydroxy acid dehydratase
pJC1	0.008
pJC1ilvD	0.050

Example 2: Construction of an *ilvA* deletion mutant of *Corynebacterium glutamicum*

5

The internal deletion of the *ilvA* gene of *Corynebacterium glutamicum* ATCC13032 was carried out using the system for gene exchange described by Schäfer *et al.* (Gene 145: 69-73 (1994)). For the construction of the inactivation vector pK19mobsacB Δ *ilvA*, first of all, an internal 241 bp BglII fragment was removed from the *ilvA* gene that was present on an EcoRI fragment in the vector pBM21. For this purpose, the vector was cut with BglII and, after separation of the *ilvA*-internal BglII fragment, religated by means of agarose gel electrophoresis. Subsequently, the incomplete gene was isolated from the vector as an EcoRI fragment and ligated into the vector pK19mobsacB linearized with EcoRI (Schäfer 1994, Gene 145: 69-73).

10

The obtained inactivation vector pK19mobsacB Δ *ilvA* was incorporated into the *E. coli* strain S 17-1 by means of transformation (Hanahan 1983, Journal of Molecular Biology 166: 557-580) and transferred to *Corynebacterium glutamicum* ATCC13032 by conjugation (Schäfer *et al.* 1990, Journal of Bacteriology 172: 1663-1666). Kanamycin-resistant clones of *Corynebacterium glutamicum* were obtained in which the inactivation vector was present in the genome in integrated form. In order to select for the excision of the vector, kanamycin-resistant clones were plated out on LB medium containing saccharose (Sambrook *et al.*, Molecular cloning. A laboratory manual (1989) Cold Spring Harbor Laboratory Press) with 15 g/l of agar, 2% glucose / 10% saccharose, and colonies were obtained that had lost the vector again through a second recombination event (Jäger *et al.* 1992, Journal of Bacteriology 174: 5462-5465).

15

By overinoculating on minimal medium plates (medium CGXII with 15 g/l of agar

20

25

(Keilhauer *et al.*, Journal of Bacteriology 175 (1993) 5595-5603) with and without 2mM L-isoleucine or with and without 50 µg/ml of kanamycin, 36 clones were isolated that were sensitive due to the excision of the vector kanamycin and that were isoleucine-auxotrophic and in which now the incomplete *ilvA* gene (Δ *ilvA* allele) was present in the genome. One strain was designated as ATCC13032 Δ *ilvA* and used further.

Example 3. Cloning the genes of pantothenate biosynthesis *panB* and *panC* from *Corynebacterium glutamicum*

Cloning the operon

Chromosomal DNA of *Corynebacterium glutamicum* ATCC13032 was isolated and cut with the restriction endonuclease *Sau3A*. After gel electrophoretic fractionation, DNA fragments within a size range of 3 kb to 7 kb or 9 kb to 20 kb were extracted and subsequently ligated into the singular *Bam*HI interface of the vector pBR322. Insert-bearing colonies were isolated on the basis of their tetracycline sensitivity after overinoculation on LB plates with 10 µg/ml of tetracycline. Through plasmid preparations (Sambrook *et al.*, Molecular cloning. A laboratory manual (1989) Cold Spring Harbor Laboratory Press) of pooled clones, 8 plasmid pools, each containing 400 plasmids having an insert size of 9 kb to 20 kb, and 9 plasmid pools, each containing 500 plasmids having an insert size of 3 kb to 7 kb, were isolated. The *E. coli* *panB* mutant SJ2 (Cronan *et al.* 1982, J. Bacteriol. 149: 916-922) was transformed with this gene library by means of electroporation (Wehrmann *et al.* 1994, Microbiology 140: 3349-3356). The transformation batches were plated out directly on CGXII medium (J. Bacteriol. (1993) 175: 5595-5603). Plasmid DNA was isolated (Sambrook *et al.* 1989) from clones that were capable of growing without pantothenate supplementation and, by means of retransformation, 8 clones were obtained whose need for

D-pantothenate was confirmed. Restriction mapping was carried out with the 8 plasmids. One of the examined vectors, referred to below as pUR1, contained an insert of 9.3 kb (Figure 3). The transformation of the *E. coli* panC mutant DV39 (Vallari *et al.* 1985, J. Bacteriol. 164: 136-142) revealed that the vector pUR1 was likewise capable of complementing the panC defect of this mutant. A 2.2-kb fragment of the insert of pUR1 was sequenced by means of the dideoxy chain termination method of Sanger *et al.* (Proceedings of the National Academy of Sciences of the United States of America U.S.A. (1977) 74: 5463-5467). The gel electrophoretic analysis was carried out with an automatic laser fluorescence sequencing device (A.L.F.) made by Amersham Pharmacia Biotech (Uppsala, Sweden). The nucleotide sequence obtained was analyzed with the HUSAR program package (Release 4.0, EMBL, Cambridge, GB). The nucleotide sequence is designated as ID SEQ NO 3. The analysis revealed the identification of two open reading frames. One open reading frame comprises 813 base pairs and has high homologies to already known panB genes from other organisms. The panB gene from *Corynebacterium glutamicum* codes for a polypeptide of 271 amino acids (see SEQ ID NO. 4). The second open reading frame comprises 837 base pairs and has high homologies to already known panC genes from other organisms. The panC gene from *Corynebacterium glutamicum* codes for a polypeptide of 271 amino acids (see SEQ ID NO. 5).

20

Example 4: Construction of a panBC deletion mutant of *Corynebacterium glutamicum*

The genomic panBC fragment of *Corynebacterium glutamicum* ATCC13032 as well as of *Corynebacterium glutamicum* ATCC13032 Δ ilvA was carried out with the system for gene exchange described by Schäfer *et al.* (Gene 145: 69-73 (1994)). For the construction of the deletion vector pK19mobsacB Δ panBC, first of all, the 3.95-kb SspI/SalI fragment was ligated with panBC with pUC18, which had previously been

cut with *Sma*I/*Sal*I. Subsequently, a 1293-bp *Eco*RV/*Nru*I fragment from the overlapping region of the *panBC* genes was removed by restriction digestion and religation. In order to allow the recloning into pK19mobsacB, the deleted *panBC* region in pUC18 was amplified with the 2 primers 5'-GAGAACTTAATCGAGCAACA-
 5 CCCCTG, 5'-GCGCCACGCCTAGCCTTGGCCCTCAA and with the polymerase chain reaction (PCR) so as to obtain a 0.5-kb Δ *panBC* fragment that carries a *Sal*I, or *Eco*RI interface at the ends. The PCR was carried out according to Sambrook *et al.* (Molecular cloning. A laboratory manual (1989) Cold Spring Harbor Laboratory Press) at an annealing temperature of 55°C [131°F]. The obtained fragment was
 10 ligated with the vector pK19mobsac that had previously been cut with *Eco*RI/*Sal*I and treated with alkaline phosphatase. The obtained inactivation vector pK19mobsacB Δ *panBC* was incorporated by means of transformation into the *Escherichia coli* strain S17-1 (Hanahan (1983) J. Mol. Biol. 166: 557-580) and transferred by means of conjugation to *Corynebacterium glutamicum* ATCC13032
 15 (Schäfer *et al.* (1990) J. Bacteriol. 172: 1663-1666). Kanamycin-resistant clones of *Corynebacterium glutamicum* were obtained in which the inactivation vector was present in the genome in integrated form. In order to select for the excision of the vector, kanamycin-resistant clones were plated out on LB medium containing saccharose (Sambrook *et al.*, Molecular cloning. A laboratory manual (1989) Cold Spring
 20 Harbor Laboratory Press) with 15 g/l of agar, 2% glucose / 10% saccharose and colonies were obtained that had lost the vector again through a second recombination event (Jäger *et al.* 1992, Journal of Bacteriology 174: 5462-5465). By overinoculating on minimal medium plates (medium CGXII with 15 g/l of agar (Keilhauer *et al.*, Journal of Bacteriology 175 (1993) 5595-5603) with and without 2mM L-isoleucine
 25 or with and without 50 µg/ml of kanamycin, 36 clones were isolated that were sensitive due to the excision of the vector kanamycin and that were isoleucine-auxotrophic and in which now the sequence of the incomplete *pan* gene (Δ *panBC* allele) was present in the genome. One strain was designated as ATCC13032 Δ *panBC*. In the same manner as was described in detail in this example, the *panBC* deletion was also intro-

duced into ATCC13032 Δ ilvA in order to obtain the strain ATCC13032 Δ ilvA- Δ panBC.

5 **Example 5: Expression of the genes ilvD, ilvBN, and ilvC in *Corynebacterium glutamicum***

The genes of acetohydroxy acid synthase (ilvBN) and of isomeroreductase (ilvC) (Cordes *et al.* 1992, Gene 112: 113-116 and Keilhauer *et al.* 1993, Journal of Bacteri-
10 ology 175: 5595-5603) and of dihydroxy acid dehydratase (ilvD) (Example 1) were cloned for expression in the vector pECM3. The vector pECM3 is a derivative of pECM2 (Jäger *et al.* 1992, Journal of Bacteriology 174: 5462-5465) that was formed through the deletion of the approximately 1-kbp long BamHI/BglII DNA fragment that carries the kanamycin-resistant gene.

15

In the pKK5 vector (Cordes *et al.* 1992, Gene 112: 113-116), the ilvBNC genes were already present in the vector pJC1 (Cremer *et al.* 1990, Molecular and General Genetics 220: 478-480) in cloned form. From this, a 5.7-kb XbaI-ilvBNC fragment was isolated and, together with a 3.1 kb-XbaI fragment of the vector pRV containing
20 the ilvD gene, it was incorporated into the vector pECM3 that had been linearized with XbaI. Here, the ligation batch was transformed into the *E. coli* strain DH5 α mc^r. The plasmid pECM3ilvBNCD was obtained from a clone.

By means of electroporation (Haynes 1989, FEMS Microbiology Letters 61: 329-
25 334) and selection for chloramphenicol resistance (3 μ g/ml), the plasmid pECM3ilvBNCD was incorporated into the strain ATCC13032 Δ ilvA and the strain ATCC13032 Δ ilvA/pECM3ilvBNCD was obtained.

Example 6: Production of L-valine with various *Corynebacterium glutamicum* strains

In order to examine their valine formation, the strains compiled in Table 4 were pre-cultivated in 60 ml of brain heart infusion medium (Difco Laboratories, Detroit, Michigan, U.S.A.) for 14 hours at 30°C [86°F]. Subsequently, the cells were washed with a 0.9% NaCl solution (w/v) and were each inoculated with this suspension of 60 ml of CgXII medium in such a way that the OD600 was 0.5. The medium was identical to the medium described by Keilhauer *et al.*, (Journal of Bacteriology (1993) 175: 5595-5603). For the cultivation of the Δ ilvA strains, however, the medium additionally received 250 mg/l of L-isoleucine. This is shown in Table 3.

Table 3

15 Composition of the CGXII medium

Component	Concentration
(NH ₄) ₂ SO ₄	20 g/l
urea	5 g/l
KH ₂ PO ₄	1 g/l
K ₂ HPO ₄	1 g/l
Mg ₂ O ₄ *7H ₂ O	0.25 g/l
3-morpholinopropane sulfonic acid	42 g/l
CaCl ₂	10 mg/l
FeSO ₄ *7H ₂ O	10 mg/l
MnSO ₄ *H ₂ O	10 mg/l
ZnSO ₄ *7H ₂ O	1 mg/l
CuSO ₄	0.2 mg/l

NiCl ₂ *6H ₂ O	0.02 mg/l
Biotin (pH 7)	0.2 mg/l
glucose	40 g/l
protocatechuic acid	0.03 mg/l

After a cultivation of 48 hours, samples were taken, the cells were centrifuged off and the supernatant was filtered under sterile conditions. The L-valine concentration of the supernatant was determined by means of high-pressure liquid chromatography with an integrated precolumn derivatization of the amino acid with o-phthdialdehyde as in Jones and Gilligan (J. Chromatogr. 266 (1983) 471-482). The results are compiled in Table 4.

10 Table 4

L-valine production with several *Corynebacterium glutamicum* strains

<i>Corynebacterium glutamicum</i>	L-valine (mM)
ATCC 13032	0.5
ATCC 13032 pJC1ilvD	2.2
ATCC 13032 pJC1ilvBNC	20.0
ATCC 13032 pJC1ilvBNCD	26.2
ATCC 13032 Δ ilvA	2.7
ATCC 13032 Δ ilvA pJC1ilvD	7.0
ATCC 13032 Δ ilvA pJC1ilvBNCD	28.5
ATCC 13032 Δ panBC	8.2
ATCC 13032 Δ ilvA Δ panBC	31.1
ATCC 13032 Δ ilvA Δ panBC pJC1ilvBNCD	72.7

Claims

1. A method for the microbial production of L-valine in which the dihydroxy
5 acid dehydratase-(ilvD)-activity and/or the ilvD-gene expression in a micro-
organism are intensified.
2. The method for the microbial production of L-valine in which the aceto-
hydroxy acid synthase-(ilvBN)-activity and isomeroreductase-(ilvC)-activity
10 and/or the ilvBNC-gene expression in a microorganism are intensified.
3. The method for the microbial production of L-valine according to Claims 1
and 2.
- 15 4. The method according to one or more of the preceding claims, **characterized
in that** the endogenous ilvD activity and/or ilvBNC activity in the micro-
organism are increased.
- 20 5. The method according to Claim 4, **characterized in that**, through mutation of
the endogenous ilvD gene and/or of the ilvBNC genes, corresponding
enzymes with a greater activity are produced.
6. The method according to one or more of the preceding claims, **characterized
in that** the ilvD gene expression and/or ilvBNC gene expression are intensi-
25 fied by increasing the number of gene copies.
7. The method according to Claim 6, **characterized in that**, in order to increase
the number of gene copies, the ilvD gene and/or the ilvBNC genes are incor-
porated into a gene construct.

8. The method according to Claim 7, **characterized in that** a microorganism is transformed with the gene construct containing the ilvD gene and/or the ilvBNC genes.
- 5
9. The method according to Claim 8, **characterized in that** *Corynebacterium glutamicum* is used as the microorganism.
10. The method according to one or more of the preceding claims, **characterized in that** a microorganism is used in which the activity of at least one enzyme participating in a metabolic pathway that reduces L-valine formation is weakened or switched off.
- 10
11. The method according to Claim 10, **characterized in that** the activity of the enzyme threonine dehydratase (ilvA) involved in the synthesis of L-isoleucine is weakened or switched off.
- 15
12. The method according to Claim 10 or 11, **characterized in that** the activity of one or more enzymes specifically involved in the synthesis of D-pantothenate is weakened or switched off.
- 20
13. The method according to Claim 12, **characterized in that** the activity of the enzyme ketopantoate hydroxymethyl transferase (panB) and/or of the enzyme pantothenate ligase (panC) is weakened or switched off.
- 25
14. A microorganism transformed with a gene construct containing the ilvD gene and/or the ilvBNC genes, in which the activity of one or more enzymes specifically involved in the synthesis of D-pantothenate is weakened or switched off.

15. The transformed microorganism according to Claim 14, in which the activity of the enzyme ketopantoate hydroxymethyl transferase (panB) and/or of the enzyme pantothenate ligase (panC) is weakened or switched off.
- 5
16. The transformed microorganism according to Claim 14 or 15, in which the activity of the enzyme threonine dehydratase (ilvA) involved in the synthesis of L-isoleucine is weakened or switched off.
- 10 17. The transformed microorganism according to one or more of Claims 14 to 16, **characterized by** *Corynebacterium glutamicum*.

Abstract

The present invention relates to a method for the microbial production of L-valine in
5 which the dihydroxy acid dehydratase-(ilvD)-activity and/or the ilvD-gene expression
in a microorganism are intensified. As an alternative to this or in combination with
this, the acetohydroxy acid synthase-(ilvBN)-activity and isomeroreductase-(ilvC)-
activity and/or the ilvBNC-gene expression in a microorganism are intensified. For
the method according to the invention, microorganisms are preferably used in which
10 the activity of at least one enzyme participating in a metabolic pathway that reduces
L-valine formation is weakened or switched off. Thus, the methods according to the
invention preferably make use of microorganisms having a defective mutation in the
threonine dehydratase-(ilvA-) gene and/or having a defective mutation in one or more
genes of the pantothenate synthesis.

15

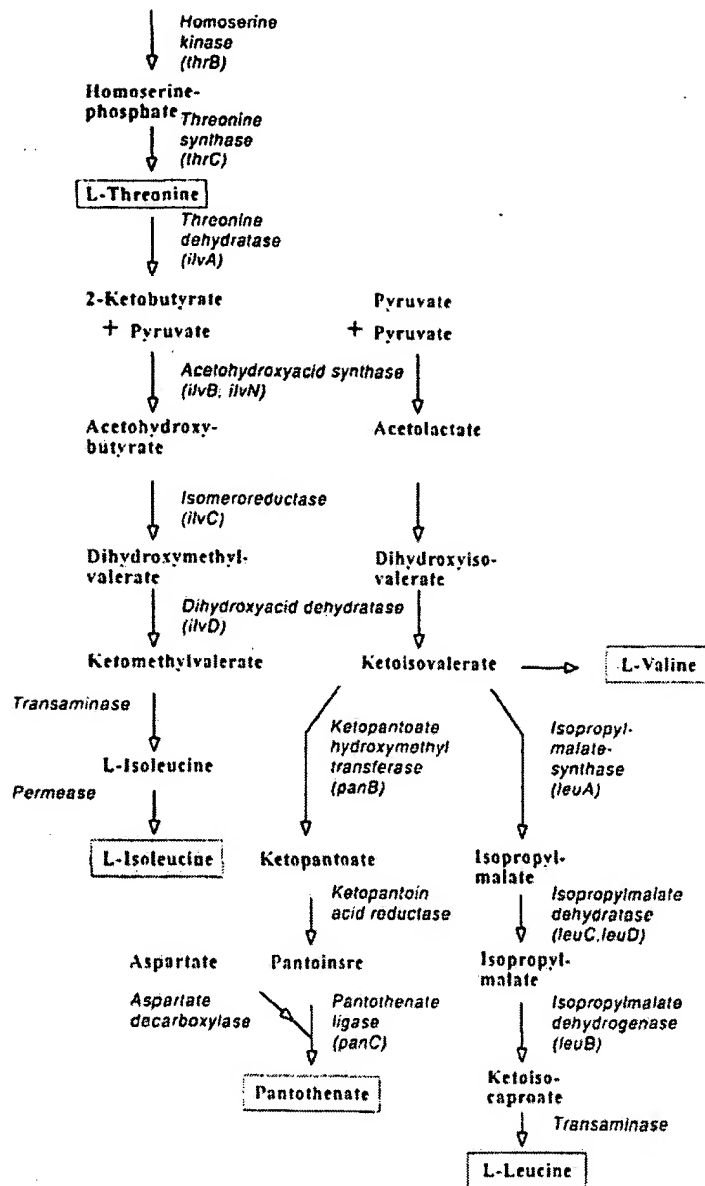


Figure 1

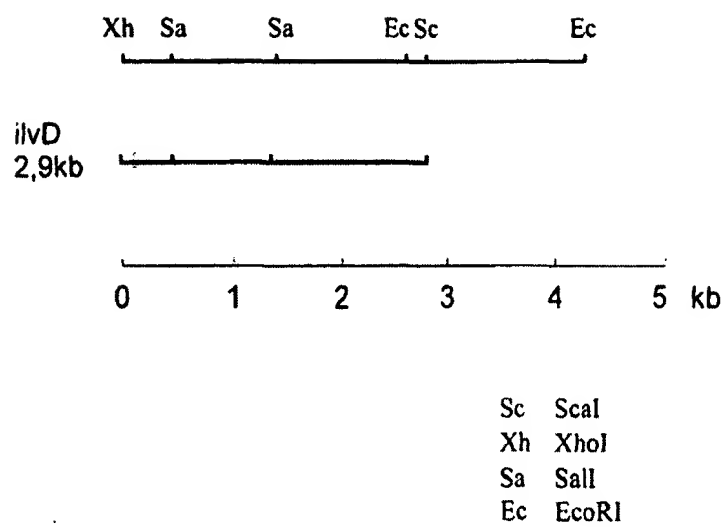


Figure 2

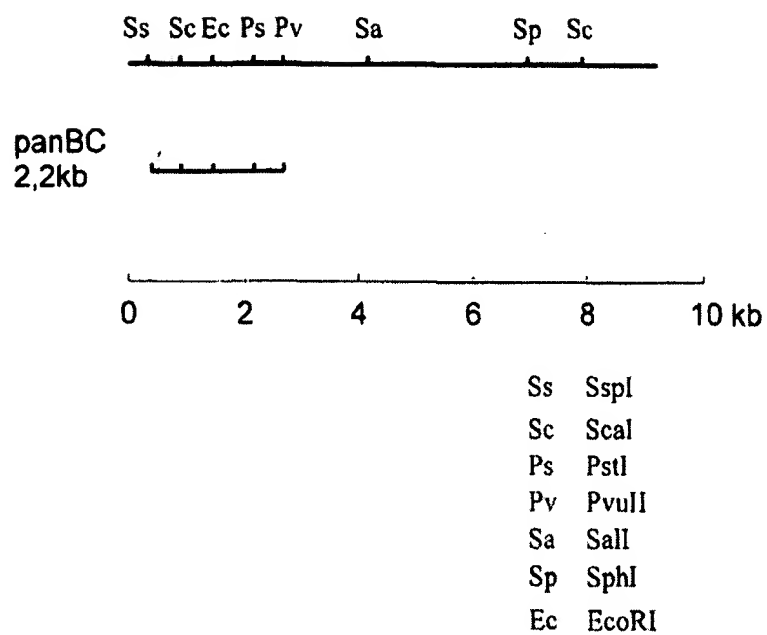


Figure 3

SEQUENCE PROTOCOL



(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Forschungszentrum Jülich GmbH
- (B) STREET: Postfach 1913
- (C) CITY: Juelich
- (E) COUNTRY: Germany
- (F) POSTAL CODE: 52425
- (G) PHONE: +49 -(0)2461-614480
- (H) FAX: +49 -(0)2461-612860

(ii) DESIGNATION OF THE INVENTION:

Valine production

(iii) NUMBER OF SEQUENCES: 5

(iv) COMPUTER-READABLE VERSION:

- (A) DATA CARRIER: floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: Patent In Release #1.0, Version #1.30 (EPA)

(2) INFORMATION ABOUT SEQ ID NO. 1:

(i) SEQUENCE DESIGNATION:

- (A) LENGTH: 2952 base pairs
- (B) TYPE: nucleotide
- (C) STRAND FORM: double strand
- (D) TOPOLOGY: linear

(ii) TYPE OF THE MOLECULE: genome DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 1:

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CTGAAACCTC ACATCGTGAT AACCTGCGT CACAGCACTA GAGTGTAATA AGCCGTCCGA
 ACCAAAGGTC CACACCTCTG CACGAGTAGA AGCTCACCCA AGTTTTCAAA GTGCCGTTGA
 TTCTTGACAA CCACCCGCCG CTCTTTAGAG CAGATTTGAA AAGCGCATCA TGATCCCACT
 TCGTTCAAAA GTCACCACCG TCGGTCGCAA TGCAGCTGGC GCTCGCGCCC TTTGGCGTGC
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2952

(2) INFORMATION ABOUT SEQ ID NO. 2:

(i) SEQUENCE DESIGNATION:

- (A) LENGTH: 612 amino acids
- (B) TYPE: amino acid
- (C) STRAND FORM: single strand
- (D) TOPOLOGY: linear

(ii) TYPE OF THE MOLECULE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 2:

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30

Gly Lys Pro Ile Val Ala Ile Val Asn Ser Tyr Thr Gln Phe
Val Pro 35                      40
45

Gly His Val His Leu Lys Asn Val Gly Asp Ile Val Ala Asp
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Arg Lys Ala Gly Gly Val Pro Lys Glu Phe Asn Thr Ile Val
Asp Asp 65                      70                      75
80

Gly Ile Ala Met Gly His Gly Gly Met Leu Tyr Ser Leu Pro
Ser Arg 85                      90
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Glu Ile Ile Ala Asp Ser Val Glu Tyr Met Val Asn Ala His
Thr Ala 100                      105
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Asp Ala Met Val Cys Ile Ser Asn Cys Asp Lys Ile Thr Pro
Gly Met 115                      120
125

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Ser Gly 130                      135                      140

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Ala His 145                      150
155                      160

Ala Pro Thr Asp Leu Ile Thr Ala Ile Ser Ala Ser Ala Ser
Asp Ala 165                      170
175

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Cys Gly Ser Cys Ser Gly Met Phe Thr Ala Asn Ser Met Asn
 Cys Leu 195 200
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 Leu Ala 210 215 220

Thr His Ala Ala Arg Arg Ala Leu Phe Glu Lys Ala Gly Glu
 Thr Val 225 230 235
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Val Glu Leu Cys Arg Arg Tyr Tyr Gly Glu Glu Asp Glu Ser
 Val Leu 245 250
 255

Pro Arg Gly Ile Ala Thr Lys Lys Ala Phe Glu Asn Ala Met
 Ala Leu 260 265
 270

Asp Met Ala Met Gly Gly Ser Thr Asn Thr Ile Leu His Ile
 Leu Ala 275 280
 285

Ala Ala Gln Glu Gly Glu Val Asp Phe Asp Leu Ala Asp Ile
 Asp Glu 290 295 300

Leu Ser Lys Asn Val Pro Cys Leu Ser Lys Val Ala Pro Asn
 Ser Asp 305 310 315
 320

Tyr His Met Glu Asp Val His Arg Ala Gly Arg Ile Pro Ala
 Leu Leu 325 330
 335

Gly Glu Leu Asn Arg Gly Gly Leu Leu Asn Lys Asp Val His
 Ser Val 340 345
 350

His Ser Asn Asp Leu Glu Gly Trp Leu Asp Asp Trp Asp Ile
 Arg Ser 355 360
 365

Gly Lys Thr Thr Glu Val Ala Thr Glu Leu Phe His Ala Ala
 Pro Gly 370 375 380

Gly Ile Arg Thr Thr Glu Ala Phe Ser Thr Glu Asn Arg Trp
 Asp Glu 385 390 395

400

Leu Asp Thr Asp Ala Ala Lys Gly Cys Ile Arg Asp Val Glu
 His Ala 405 410
 415

Tyr Thr Ala Asp Gly Gly Leu Val Val Leu Arg Gly Asn Ile
 Ser Pro 420 425
 430

Asp Gly Ala Val Ile Lys Ser Ala Gly Ile Glu Glu Glu Leu
 Trp Asn 435 440
 445

Phe Thr Gly Pro Ala Arg Val Val Glu Ser Gln Glu Glu Ala
 Val Ser 450 455 460

Val Ile Leu Thr Lys Thr Ile Gln Ala Gly Glu Val Leu Val
 Val Arg 465 470 475
 480

Tyr Glu Gly Pro Ser Gly Gly Pro Gly Met Gln Glu Met Leu
 His Pro 485 490
 495

Thr Ala Phe Leu Lys Gly Ser Gly Leu Gly Lys Lys Cys Ala
 Leu Ile 500 505
 510

Thr Asp Gly Arg Phe Ser Gly Gly Ser Ser Gly Leu Ser Ile
 Gly His 515 520
 525

Val Ser Pro Glu Ala Ala His Gly Gly Val Ile Gly Leu Ile
 Glu Asn 530 535 540

Gly Asp Ile Val Ser Ile Asp Val His Asn Arg Lys Leu Glu
 Val Gln 545 550
 555 560

Val Ser Asp Glu Glu Leu Gln Arg Arg Arg Asp Ala Met Asn
 Ala Ser 565 570
 575

Glu Lys Pro Trp Gln Pro Val Asn Arg Asn Arg Val Val Thr
 Lys Ala 580 585
 590

Leu Arg Ala Tyr Ala Lys Met Ala Thr Ser Ala Asp Lys Gly
 Ala Val 595 600
 605

Arg Gln Val Asp
610

(2) INFORMATION ABOUT SEQ ID NO. 3:

(i) SEQUENCE DESIGNATION:
(A) LENGTH: 2164 base pairs
(B) TYPE: nucleotide
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF THE MOLECULE: genome DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 3:

GCTTCGGGGT ACCAATTCCT TTAAGAACCA TCAGATCAAT CTGTTGTACA TTCTCGGCCA
GATTCAGCTT TTCGGTAAGG ACGAAACACT TTCACTTGAA TCGGCAGCAA AGTTTCTTAA
AGTTTCTAAG GCAACTGCAA CGAGGTATTT TAGAACTCTC CGAGAAATGG AATTAGTTCA
CGAGGTCAGC AAACGCCCTT TGCGGTTTGC GCTCACGGAT AAAGGTCGTG AGATAGTAGG
TCTTGAGGTA AAAATTTGAC TCCATAACGA GAACTTAATC GAGCAACACC CCTGAACAGT
GAATCAAATC GGAATTTATT TATTCTGAGC TGGTCATCAC ATCTATACTC ATGCCCATGT
CAGGCATTGA TGCAAAGAAA ATCCGCACCC GTCATTTCCG CGAAGCTAAA GTAAACGGCC
AGAAAGTTTC GGTTCCTACC AGCTATGATG CGCTTTCGGC GCGCATTTTTT GATGAGGCTG
GCGTCGATAT GCTCCTTGTT GGTGATTCCG CTGCCAACGT TGTGCTGGGT CGCGATACCA
CCTTGTCGAT CACCTTGAT GAGATGATTG TGCTGGCCAA GGCGGTGACG ATCGCTACGA
AGCGTGCGCT TGTGGTGTT GATCTGCCGT TTGGTACCTA TGAGGTGAGC CCAAATCAGG
CGGTGGAGTC CGCGATCCGG GTCATGCGTG AAACGGGTGC GGCTGCGGTG AAGATCGAGG
GTGGCGTGGA GATCGCGCAG ACGATTCGAC GCATTGTTGA TGCTGGAATT CCGGTTGTCTG
GCCACATCGG GTACACCCCG CAGTCCGAGC ATTCTTGGG CGGCCACGTG GTTCAGGGTC
GTGGCGCGAG TTCTGGAAAG CTCATCGCCG ATGCCCGCGC GTTGGAGCAG GCGGGTGCGT
TTGCGGTTGT GTTGAGATG GTTCCAGCAG AGGCAGCGCG CGAGGTTACC GAGGATCTTT
CCATCACCAC TATCGGAATC GGTGCCGGCA ATGGCACAGA TGGGCAGGTT TTGGTGTGGC

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AGGATGCCTT CGGCCTCAAC CGCGGCAAGA AGCCACGCTT CGTCCGCGAG TACGCCACCT 1
TGGGCGATTG CTTGCACGAC GCCGCGCAGG CCTACATCGC CGATATCCAC GCGGGTACCT 1
TCCCAGGCGA AGCGGAGTCC TTTTAATGCA GGTAGCAACC ACAAAGCAGG CGCTTATCGA 1
CGCCCTCCTC CACCACAAAT CCGTCGGGCT CGTCCCCACC ATGGGTGCGC TACACAGCGG 1
ACACGCCTCG TTGGTTAAAG CAGCACGCGC TGAAAACGAC ACTGTTGTAG CCAGTATTTT 1
TGTCAATCCC CTGCAGTTTG AAGCACTCGG TGATTGCGAT GATTACCGCA ACTATCCCCG 1
CCAACTCGAC GCCGATTTAG CACTGCTTGA AGAGGCAGGT GTGGATATTG TGTTCGCACC 1
CGATGTGGAG GAAATGTACC CCGGTGGCTT GCCACTAGTG TGGGCGCGCA CCGGTTCCAT 1
CGGAACAAAA TTGGAGGGTG CCAGCAGGCC TGGCCATTTC GATGGTGTGG CTACCGTGGT 1
GGCGAAGCTG TTCAATTGCG TCGGCCCTGA TCGTGCATAT TTTGGACAAA AAGATGCTCA 1
GCAGGTGCGG GTGATTCGGC GATTGGTTGC CGATCTAGAC ATTCCCGTGG AGATTGCTCC 1
CGTTCCGATT ATTCGTGGCG CCGATGGCTT AGCCGAATCC AGCCGCAATC AACGTCTTTC 1
TGCGGATCAG CGAGCGCAAG CTCTGGTGCT GCCGCAGGTG TTGAGTGGGT TGCAGCGTCG 1
AAAAGCAGCT GGTGAAGCGC TAGATATCCA AGGTGCGCGC GACACCTTGG CCAGCGCCGA 1
CGGCGTGCGC TTGGATCACC TGGAAATGTG CGATCCAGCC ACCCTCGAAC CATTAGAAAT 1
CGACGGCCTG CTCACCCAAC CAGCGTTGGT GGTGGGCGCG ATTTTCGTGG GGCCGGTGCG 1
GTTGATCGAC AATATCGAGC TCTAGTACCA ACCCTGCGTT GCAGCACGCA GCTTCGCATA 2
ACGCGTGCTC AGCTCAGTGT TTTTAGGTGC GCGGTGCGGA TCGGAACCGG GAGTTGGCCA 2
CTGCGGTGGC GTGGCCTCAC CCGACAGCGC CCATGCCGCC TGACGAGCTG CACCCAACGC 2
CACA 2

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(2) INFORMATION ABOUT SEQ ID NO. 4:

(i) SEQUENCE DESIGNATION:

- (A) LENGTH: 271 amino acids
- (B) TYPE: amino acid
- (C) STRAND FORM: single strand
- (D) TOPOLOGY: linear

(ii) TYPE OF THE MOLECULE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 4:

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Met Pro Met Ser Gly Ile Asp Ala Lys Lys Ile Arg Thr Arg
His Phe 1

Arg Glu Ala Lys Val Asn Gly Gln Lys Val Ser Val Leu Thr
Ser Tyr

          20          25          30

Asp Ala Leu Ser Ala Arg Ile Phe Asp Glu Ala Gly Val Asp
Met Leu 35          40
45

Leu Val Gly Asp Ser Ala Ala Asn Val Val Leu Gly Arg Asp
Thr Thr 50          55          60

Leu Ser Ile Thr Leu Asp Glu Met Ile Val Leu Ala Lys Ala
Val Thr 65          70          75
80

Ile Ala Thr Lys Arg Ala Leu Val Val Val Asp Leu Pro Phe
Gly Thr 85          90
95

Tyr Glu Val Ser Pro Asn Gln Ala Val Glu Ser Ala Ile Arg
Val Met 100          105
110

Arg Glu Thr Gly Ala Ala Ala Val Lys Ile Glu Gly Gly Val
Glu Ile 115          120
125

Ala Gln Thr Ile Arg Arg Ile Val Asp Ala Gly Ile Pro Val
Val Gly 130          135          140

His Ile Gly Tyr Thr Pro Gln Ser Glu His Ser Leu Gly Gly
His Val 145          150
155          160

Val Gln Gly Arg Gly Ala Ser Ser Gly Lys Leu Ile Ala Asp
Ala Arg 165          170
175

Ala Leu Glu Gln Ala Gly Ala Phe Ala Val Val Leu Glu Met
Val Pro 180          185
190

Ala Glu Ala Ala Arg Glu Val Thr Glu Asp Leu Ser Ile Thr
Thr Ile 195          200
205

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Gly Ile Gly Ala Gly Asn Gly Thr Asp Gly Gln Val Leu Val
 Trp Gln 210 215 220

Asp Ala Phe Gly Leu Asn Arg Gly Lys Lys Pro Arg Phe Val
 Arg Glu 225 230 235 240

Tyr Ala Thr Leu Gly Asp Ser Leu His Asp Ala Ala Gln Ala
 Tyr Ile 245 250 255

Ala Asp Ile His Ala Gly Thr Phe Pro Gly Glu Ala Glu Ser
 Phe 260 265 270

(2) INFORMATION ABOUT SEQ ID NO. 5:

- (i) SEQUENCE DESIGNATION:
 (A) LENGTH: 279 amino acids
 (B) TYPE: amino acid
 (C) STRAND FORM: single strand
 (D) TOPOLOGY: linear

(ii) TYPE OF THE MOLECULE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 5:

Met Gln Val Ala Thr Thr Lys Gln Ala Leu Ile Asp Ala Leu
 Leu His 1 5 10
 15

His Lys Ser Val Gly Leu Val Pro Thr Met Gly Ala Leu His
 Ser Gly 20 25
 30

His Ala Ser Leu Val Lys Ala Ala Arg Ala Glu Asn Asp Thr
 Val Val 35 40
 45

Ala Ser Ile Phe Val Asn Pro Leu Gln Phe Glu Ala Leu Gly
 Asp Cys 50 55 60

Asp Asp Tyr Arg Asn Tyr Pro Arg Gln Leu Asp Ala Asp Leu
 Ala Leu 65 70 75

80

Leu Glu Glu Ala Gly Val Asp Ile Val Phe Ala Pro Asp Val
 Glu Glu 85 90
 95

Met Tyr Pro Gly Gly Leu Pro Leu Val Trp Ala Arg Thr Gly
 Ser Ile 100 105
 110

Gly Thr Lys Leu Glu Gly Ala Ser Arg Pro Gly His Phe Asp
 Gly Val 115 120
 125

Ala Thr Val Val Ala Lys Leu Phe Asn Leu Val Arg Pro Asp
 Arg Ala 130 135 140

Tyr Phe Gly Gln Lys Asp Ala Gln Gln Val Ala Val Ile Arg
 Arg Leu 145 150 155
 160

Val Ala Asp Leu Asp Ile Pro Val Glu Ile Arg Pro Val Pro
 Ile Ile 165 170
 175

Arg Gly Ala Asp Gly Leu Ala Glu Ser Ser Arg Asn Gln Arg
 Leu Ser 180 185
 190

Ala Asp Gln Arg Ala Gln Ala Leu Val Leu Pro Gln Val Leu
 Ser Gly 195 200
 205

Leu Gln Arg Arg Lys Ala Ala Gly Glu Ala Leu Asp Ile Gln
 Gly Ala 210 215 220

Arg Asp Thr Leu Ala Ser Ala Asp Gly Val Arg Leu Asp His
 Leu Glu 225 230 235
 240

Ile Val Asp Pro Ala Thr Leu Glu Pro Leu Glu Ile Asp Gly
 Leu Leu 245 250
 255

Thr Gln Pro Ala Leu Val Val Gly Ala Ile Phe Val Gly Pro
 Val Arg 260 265
 270

Leu Ile Asp Asn Ile Glu Leu
 275



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